INVITED REVIEW

Vaccination of chickens with recombinant *Salmonella* expressing M2e and CD154 epitopes increases protection and decreases viral shedding after low pathogenic avian influenza challenge[1]

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ABSTRACT Avian influenza (AI) is a significant public health concern and serious economic threat to the commercial poultry industry worldwide. Previous research demonstrates that antibodies against M2e confer protection against influenza challenge. Using the Red recombinase system in combination with overlapping extension PCR, we recently developed several novel attenuated *Salmonella* Enteritidis strains that express a protective M2e epitope in combination with a potential immune-enhancing CD154 peptide sequence on the *Salmonella* outer membrane protein lamB. Commercial Leghorn chicks were orally immunized (immunization dose: 10^6 to 10^8 cfu/chick) with saline (negative control) or one of the recombinant *Salmonella* strains [Δ*aro*A M2e-CD154, Δ*htr*A M2e-CD154, Δ*aro*A-Δ*htr*A M2e(4)-CD154] on day of hatch and 21 d posthatch. These candidate vaccine strains were evaluated for their ability to invade, colonize, and persist in tissues and elicit an M2e-specific antibody response. The vaccine candidate strain Δ*aro*A M2e-CD154 exhibited significantly greater organ invasion in the liver and spleen at d 7 ($P > 0.05$); however, no marked differences in colonization of the cecal tonsils were observed. Vaccinated chickens exhibited significantly increased M2e-specific IgG responses, which were further enhanced by simultaneous expression of CD154 ($P < 0.05$). Virus neutralization assays gave neutralizing indices of 6.6, 6.3, and 6.3 for Δ*aro*A M2e-CD154, Δ*htr*A M2e-CD154, and Δ*aro*A-Δ*htr*A M2e(4)-CD154 seven days post booster immunization, respectively, indicating effective neutralization of AI by serum IgG of vaccinated chickens. In a subsequent direct challenge study, specific-pathogen-free Leghorn chicks immunized with Δ*aro*A-Δ*htr*A M2e(4)-CD154 offered significant protection against direct challenge with low pathogenic AI H7N2, but not highly pathogenic H5N1 AI. Taken together, these data suggest that these *Salmonella*-vectored vaccines expressing M2e in association with CD154 are effective at protecting chickens against low pathogenic AI.

Key words: *Salmonella*, M2e, vaccine, avian influenza

2009 Poultry Science 88:2244–2252
doi:10.3382/ps.2009-00251

INTRODUCTION

Highly pathogenic avian influenza (**HPAI**) is a significant public health concern and a serious economic threat to the commercial poultry industry worldwide. In the last 5 yr, there has been a substantial increase in the number of HPAI outbreaks in poultry flocks and the number of countries reporting outbreaks continues to increase (Capua and Alexander, 2004; Zhao et al., 2005; Capua and Marangon, 2006). Increasing the resistance of the poultry population against avian influenza (**AI**) will not only prevent substantial economic losses to the poultry industry due to the high morbidity and mortality associated with AI in poultry flocks but will also reduce the significant health risk for the human population by reducing shed and thereby transmission.

Vaccination is widely considered an effective means to prevent infectious diseases, but until recently, the vaccination of poultry against AI has not been widely recommended (Capua and Marangon, 2004; Zhao et al., 2005; OIE/FAO, 2005; Huber et al., 2006; Swayne and Kapczynski, 2008a). Current influenza vaccines target

Received May 25, 2009.
Accepted August 4, 2009.
[1]Presented as part of the Poultry Science Association Keynote Symposium, "Avian Influenza: Vectors, Vaccines, Public Health, and Product Marketability," July 20, 2008, at the Poultry Science Association meeting, Niagara Falls, Ontario, Canada..
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antibody production against the surface glycoproteins hemagglutinin (**HA**) and neuraminidase (Zharikova et al., 2005; Swayne and Kapczynski, 2008a). However, these antigenic molecules are highly susceptible to recombination and mutations (Steinhauer and Skehel, 2002; Fiers et al., 2004). This results in the need to frequently update the vaccine to protect against currently circulating strains. Therefore, there is a critical need for new influenza vaccines, which are able to provide protective immunity against current and future AI virus strains, and for poultry vaccines that can be cost-effectively amplified and delivered.

Similar to HA and neuraminidase, the M2 protein is an integral membrane protein of influenza A viruses (Pinto et al., 1992). It is well documented that the external domain of the M2 protein (M2e) is highly conserved among human influenza type A viruses and M2e-specific antibodies have the potential to provide a broad protective immunity across influenza A strains (Neirynck et al., 1999; Mozdzanowska et al., 2003; Fiers et al., 2004; Zou et al., 2004). Although natural infection and current influenza vaccines do not appear to elicit a strong M2e-specific antibody response, presenting M2e on a suitable carrier greatly enhances its immunogenecity (Neirynck et al., 1999; Fiers et al., 2004; De Filette et al., 2005). In addition, M2e specificity has been shown to decrease the infectivity, morbidity, and mortality associated with influenza infection and provides significant protection against subsequent influenza challenge in several animal models (Neirynck et al., 1999; Fan et al., 2004; Liu et al., 2004, 2005; Zharikova et al., 2005; Ernst et al., 2006). Therefore, M2e is considered a potential vaccine candidate for inducing cross-reactive protection against influenza type A viruses (Neirynck et al., 1999; Fiers et al., 2004).

A member of the tumor necrosis factor ligand family, CD154 (CD40L), is expressed primarily on the surface of activated T-cells and plays several key roles in the regulation of cellular immune responses (Xu and Song, 2004; Tregaskes et al., 2005). Studies have demonstrated that the CD40-CD154 interaction can upregulate co-stimulatory molecules, activate antigen-presenting cells, and influence T-cell-mediated effector functions (Grewal and Flavell, 1998; Miga et al., 2000). The CD40 antibodies mimicking the endogenous CD40-CD154 interaction have been shown to exhibit potent adjuvant effects when attached to antigens (Barr et al., 2003). In addition, anti-CD40 antibodies attached to a synthetic peptide vaccine candidate have induced protective immunity against influenza A virus in mice (Ninomiya et al., 2002).

Oral live attenuated *Salmonella* vaccine vectors expressing recombinant foreign antigens have previously been shown to stimulate systemic, mucosal, humoral, and cell-mediated immune responses against *Salmonella* and the foreign antigens (Mollenkopf et al., 2001; Kotton and Hohmann, 2004; Ashby et al., 2005). Vaccine vectors that elicit mucosal immune responses against multiple subtypes of influenza and can be modified quickly as epitopes continue to evolve present a promising alternative approach compared with existing vaccine technologies. *Salmonella* vectors also have the potential advantage of being extremely inexpensive to amplify-manufacture and because they do not have to be injected and can be administered by spray or drinking water, they are much more acceptable for widespread administration to commercial poultry. Therefore, the objective of this study was to construct and evaluate several recombinant live attenuated *Salmonella* vaccine vectors expressing multiple copies of an M2e epitope, alone or in combination with CD154 (CD40L), for their ability to stimulate systemic and humoral responses against M2e and protect against direct AI challenge.

MATERIALS AND METHODS

Attenuation of Salmonella Vaccine Candidate Strains

Multiple strains of *Salmonella* Enteritidis were attenuated by introducing defined, irreversible deletion mutations of the *aro*A or *htr*A gene, or both, of the *Salmonella* Enteritidis genome as described previously (Husseiny and Hensel, 2005). Briefly, the target gene sequence in the bacterial genome of *Salmonella* Enteritidis was replaced with the kanamycin-resistant ($\mathbf{Km^R}$) gene sequence. This was performed using 3S-PCR and electroporation of the 3S-PCR products into electrocompetent *Salmonella* cells containing the pKD46 plasmid. The resulting cell mixture was plated on Luria-Bertani (**LB**) agar plates supplemented with kanamycin (**Km**) to select for positive clones containing the Km^R gene. The Km^R gene was inserted into the genomic region containing the genes of interest (*aro*A or *htr*A) by flanking the Km^R gene with sequences homologous to the genes of interest. Once Km^R mutants were obtained, the deletion mutations were confirmed by PCR and DNA sequencing (data not shown).

Construction of Recombinant M2e-CD154 Inserts

Recombinant *Salmonella* Enteritidis strains containing stable integrated copies of a codon-optimized M2e-CD154 insert (EVETPIRN-WAEKGYYTMS) were constructed using the method of Cox et al. (2007). Briefly, an I-SceI enzyme site along with the Km^R gene were introduced into loop 9 of the lamB gene by design of a PCR product, which had the I-SceI enzyme site and Km^R gene flanked by approximately 200 to 300 bp of DNA on each side, homologous to the up- and downstream regions of loop 9. The PCR product was electroporated into electrocompetent attenuated *Salmonella* cells containing the pKD46 plasmid and the resulting cell mixture was plated on LB agar plates supplemented with Km to select for positive clones con-

taining a Km^R gene. After the Sce-I-Km mutation was made in loop 9, this region was replaced by a codon-optimized M2e-CD154 DNA sequence (Burns and Beacham, 1985; Vega et al., 2003; Fiers et al., 2004). This second 3S-PCR reaction produced a M2e-CD154 insert flanked by loop 9 up- and downstream regions, and the resulting PCR product was electroporated into electrocompetent attenuated *Salmonella* cells containing the Sce-I-Km mutation described above. Plasmid pBC-I-SceI was also electroporated into the cells along with the insert because the plasmid produces the I-SceI enzyme, which recognizes and cleaves a sequence creating a gap at the I-SceI enzyme site in the loop 9 region of the lamB gene where the M2e-CD154 sequence was inserted into the *Salmonella* Enteritidis genome (Kang et al., 2004). The plasmid also carries with it a chloramphenicol-resistant gene as the inserts that replace the Km^R gene the mutations must have a new selection marker to counterselect against the previous I-SceI-Km mutation. After electroporation, cells were plated on LB agar plates containing chloramphenicol at 25 μg/mL for the selection of positive mutants. Once positive mutation-inserts were suspected, PCR and DNA sequencing were performed to confirm that the insertion sequences were present and correct (data not shown).

Cell surface expression of the recombinant inserts was confirmed with a simple (+/−) agglutination test using antisera generated against a synthetic M2e peptide (Genscript Corp., Piscataway, NJ) in Leghorn hens as a positive control (data not shown).

Immunogenicity of M2e-CD154 Constructs In Vivo

Two hundred twenty day-of-hatch Leghorn chicks were obtained from a local commercial hatchery and randomly assigned to 1 of 4 treatment groups: saline only (negative control), *Salmonella* Enteriditis 13A Δ*aro*A M2e-CD154 (Δ*aro*A M2e-CD154), *Salmonella* Enteriditis 13A Δ*htr*A M2e-CD154 (Δ*htr*A M2e-CD154), *Salmonella* Enteriditis 13A Δ*aro*A-Δ*htr*A M2e(4)-CD154 [Δ*aro*A-Δ*htr*A M2e(4)-CD154; n = 55/pen]. Each treatment group was housed in an individual floor pen on fresh pine litter and provided water and feed ad libitum. On day of hatch, all chicks in each treatment group were inoculated, via oral gavage, with 0.25 mL of a suspension containing approximately 10^6 to 10^8 cfu/mL of the appropriate treatment. On d 7, 21 (before booster inoculation), and 28 posthatch, 10 to 12 birds from each treatment group were humanely killed and their liver, spleen, and cecal tonsils were aseptically removed for the determination of organ invasion, colonization, and clearance of the *Salmonella* vaccine vector strains. In addition, blood samples were collected from 15 birds per treatment group and the serum was used for determining M2e antibody response and virus neutralization (**VN**) on d 21, 28, 35, and 42 posthatch.

Organ Invasion, Colonization, and Clearance of Salmonella Vaccine Vectors

The tissue samples were placed in 15.0 mL of tetrathionate broth and incubated for 24 h at 37°C. The liver and spleen of each bird were pooled and assayed as 1 sample. After incubation, 0.1 mL of the enriched sample suspension was streaked onto brilliant green agar plates and incubated; characteristic colonies were confirmed as *Salmonella* by observation of typical colony morphology.

Positive *Salmonella* isolates recovered from the birds in each treatment group were subjected to analysis by PCR to ensure that the strain originally given to the birds was equivalent to the strain recovered. In each treatment group, PCR confirmed that the recovered strains were the same as the challenge strains.

Viruses

The influenza viruses used in the direct challenge AI study were A/Turkey/Virginia/158512/2002 H7N2 low pathogenic AI (**LPAI**) and A/Egret/Hong Kong/757.2/2002 H5N1 HPAI. Viruses were grown and tittered in 9- to 11-d-old embryonated specific-pathogen-free (**SPF**) chicken eggs as described previously (Swayne et al., 1998).

Measurement of M2e Antibody Response

Blood samples were collected in serum separating syringes (Sarstedt Inc., Newton, NC) on 21, 28, 35, and 42 d posthatch and stored at room temperature overnight. Once serum was collected, samples were stored at −80°C until assayed in an antigen capture ELISA to determine relative M2e antibody responses. Briefly, individual wells of a 96-well plate (Nunc/Thermo Fisher Scientific, Rochester, NY) were coated with 5 μg/mL of M2e conjugated to keyhole limpet hemocyanin (GenScript Corp.). Antigen adhesion was allowed to proceed at 4°C overnight. Plates were rinsed with 2% Tween and 1× PBS and blocked with 2% albumin from bovine serum, 0.5% gelatin, 0.5% casein, and 1× PBS solution. Plates were incubated at 4°C overnight. After incubation, plates were emptied and incubated for 2 h at room temperature with the previously collected sera in a 1:99 ratio of serum to a 1× PBS solution containing 2% fetal calf serum. Each plate contained a positive control (M2e antiserum) and a negative control (no added sera) for plate comparison. The plates were rinsed again with the previously described rinsing solution, followed by incubation of a secondary antibody (peroxidase conjugated goat anti-chicken IgY; Kirkegaard and Perry Laboratories, Gaithersburg, MD) for an additional hour at room temperature in a 1:10,000 ratio of secondary antibody to a 1× PBS solution containing 2% fetal

calf serum. After subsequent rinsing, the plates were developed using tetramethylbenzidine substrate (BD Biosciences, San Diego, CA), a peroxidase substrate kit (BD Biosciences), and absorbances were read on a spectrophotometer at 450 nm. The absorbance obtained for the positive control, negative control, and experimental samples was used to calculate sample:positive control ratios (**S/P**; Brown et al., 1991; Davies et al., 2003) using the following calculation: (sample mean − negative control mean)/(positive control mean − negative control mean).

VN Assay

For the VN assay, serum samples from each treatment group on d 21, 28, 35, and 42 posthatch were pooled and sent to an independent laboratory for analysis (Charles River Laboratories Inc., Wilmington, MA). The reference strain used in the assay was A/Turkey/Wisconsin/1966 H9N2 LPAI. Serum samples were diluted 2-fold and the VN endpoint was determined as the last dilution with complete inhibition of cytotoxicity. The VN index is expressed as the reciprocal dilution ($\log_2$) of serum inhibiting cytopathogenic effect in cell culture.

Direct AI Challenge Study

Forty day-of-hatch commercial SPF Leghorn chicks were randomly assigned to 1 of 2 treatment groups: saline only (negative control) or *Salmonella* Enteritidis 13A Δ*aro*A-Δ*htr*A M2e(4)-CD154 [Δ*aro*A-Δ*htr*A M2e(4)-CD154; n = 20/group]. Each treatment group was housed in an individual floor pen on fresh pine litter and provided water and feed ad libitum. On day of hatch, the chicks in each treatment group were inoculated, via oral gavage, with 0.25 mL of saline (negative control) or 0.25 mL of a suspension containing 10^6 to 10^8 cfu/mL of the candidate vaccine vector strain [Δ*aro*A-Δ*htr*A M2e(4)-CD154]. On d 21 posthatch, the birds in each treatment group were given a booster inoculation, via oral gavage, with 0.25 mL of saline (negative control) or 0.25 mL of a suspension containing 10^6 to 10^8 cfu/mL of the candidate vaccine vector strain [Δ*aro*A-Δ*htr*A M2e(4)-CD154]. On d 35 posthatch, the birds were transferred into negative-pressure stainless steel Horsfall units containing high-efficiency particulate air filters in a USDA-certified biosafety level 3 enhanced facility for LPAI or HPAI challenge. Three weeks after the booster inoculation (42 d posthatch), 10 birds from each treatment group were challenged intranasally with 10^6 50% embryo infectious dose/bird of A/Turkey/Virginia/158512/2002 H7N2 LPAI, whereas the remaining 10 birds were challenged intranasally with 10^5 50% embryo infectious dose A/Egret/Hong Kong/757.2/2002 H5N1 HPAI per bird. After AI challenge, birds were monitored daily for morbidity and mortality for 14 d. Birds displaying severe clinical signs of disease were euthanized by overdose of sodium pentobarbital. To determine the incidence of viral shedding, oral and cloacal swabs were taken on d 2 and 4 postchallenge.

Virus Detection

Virus detection from oral and cloacal swabs on d 2 and 4 post-AI challenge was performed as described previously (Tumpey et al., 2004). Briefly, swabs were collected into 2 mL of brain-heart infusion broth with antibiotics (1,000 units/mL of penicillin G, 200 μg/mL of gentamicin sulfate, and 4 μg/mL of amphotericin B; Sigma Chemical Company, St. Louis, MO) from each bird on d 0, 2, and 4 postchallenge and 0.2 mL was injected in 9- to 11-d-old embryonated SPF chicken eggs. The inoculated eggs were incubated at 37°C for 72 to 96 h and allantoic fluid was harvested and screened for the presence of AI by the HA test following standard procedures (Swayne et al., 1998).

Statistical Analysis

Data from the immunogenicity study were subjected to ANOVA using the GLM procedure of SAS (SAS Institute Inc., Cary, NC) and treatment means were partitioned by LSMEANS analysis. Data from the direct AI challenge study were subjected to the Mantel-Cox log-rank test. A probability of $P < 0.05$ was required for statistical significance.

RESULTS

Vaccine Vector Organ Invasion, Colonization, and Clearance

Before the immunization studies, *Salmonella* Enteritidis phage type 13A was selected as the parent strain for all candidate vaccine strains due to its ability to invade and persist in young chicks (data not shown).

After immunization of day-of-hatch chicks, the Δ*aro*A M2e(4)-CD154 vaccine strain exhibited significantly greater ($P < 0.05$) organ invasion in the liver and spleen on d 7 posthatch when compared with the other candidate vaccine strains and the negative control (Table 1). In contrast, there were no marked differences in organ invasion by d 21 or 28 posthatch. The 3 recombinant *Salmonella* candidate vaccine strains were able to effectively colonize the cecal tonsils when compared with the negative control group ($P < 0.05$). The birds were shown to clear the vaccine strains from the liver, spleen, and cecal tonsils by d 21 posthatch. However, all 3 candidate vaccine strains were reisolated in the cecal tonsils on d 28 posthatch. *Salmonella* was not detected in any of the negative control birds at any time point throughout the study.

Table 1. Organ invasion, colonization, and clearance of attenuated recombinant *Salmonella* vaccine vectors expressing M2e-CD154 after oral immunization in commercial Leghorn chicks[1]

	Liver-spleen			Cecal tonsils		
Treatment	7 d	21 d	28 d	7 d	21 d	28 d
Negative control	$0/12^{a}$	$0/12^{a}$	$0/12^{a}$	$0/12^{a}$	$0/12^{a}$	$0/12^{a}$
Δ*aro*A M2e-CD154	$7/12^{b}$	$0/12^{a}$	$0/12^{a}$	$12/12^{b}$	$0/12^{a}$	$1/12^{a}$
Δ*htr*A M2e-CD154	$1/12^{a}$	$0/12^{a}$	$0/12^{a}$	$11/12^{b}$	$0/12^{a}$	$2/12^{a}$
Δ*aro*A-*htr*A M2e-CD154	$0/11^{a}$	$0/12^{a}$	$0/10^{a}$	$11/11^{b}$	$0/12^{a}$	$1/10^{a}$

[a,b]Means with no common superscript within columns differ significantly ($P < 0.05$).

[1]Incidence of the attenuated recombinant *Salmonella* vaccine vector is represented as the number of positive liver, spleen, or cecal tonsils out of 10 to 12 birds. Chicks were orally inoculated with approximately 10^6 to 10^8 cfu of the appropriate treatment on day of hatch and 21 d posthatch. On d 7, 21 (before booster immunization), and 28 posthatch, 10 to 12 birds from each treatment group were euthanatized, and the livers, spleens, and ceca tonsils were collected for the determination (+/−) of the attenuated recombinant *Salmonella* vaccine vector. The liver and spleen of each bird were pooled and assayed as 1 sample.

M2e Serum Antibody Response

To test for the presence or absence of M2e-specific antibodies, sera were collected from experimentally vaccinated chickens and tested in a M2e ELISA. Serum from 15 saline-vaccinated chickens and 45 recombinant *Salmonella*-M2e-vaccinated (15/group) chickens were tested at d 21, 28, 35, and 42 posthatch. The birds vaccinated with recombinant *Salmonella* exhibited a significantly higher M2e-specific IgG antibody response, in terms of S/P ratios, compared with the birds given saline only (Figure 1). The S/P ratios were greater than 0.2 on all days tested for all birds receiving *Salmonella* expressing M2e-CD154. For each of the 3 *Salmonella* vaccine strains, the antibody titers were significantly highest 2 wk after the booster immunization (d 35 posthatch). No antibodies were detected in chickens receiving oral vaccination with saline.

VN

To determine the functional characteristics of antibodies produced against the M2e protein, in vitro VN testing of serum from saline and recombinant *Salmonella*-M2e-vaccinated chickens was performed. Serum samples were pooled from each group and tested for the ability to neutralize H9N2 LPAI in cell culture. Before the booster immunization on d 21 posthatch, VN assays gave neutralizing indexes of 5.8, 6, and 5.8 for the Δ*aro*A M2e-CD154, Δ*htr*A M2e-CD154, and *aro*A-Δ*htr*A M2e(4)-CD154 strains, respectively (Table 2). After the booster immunization, neutralizing index-

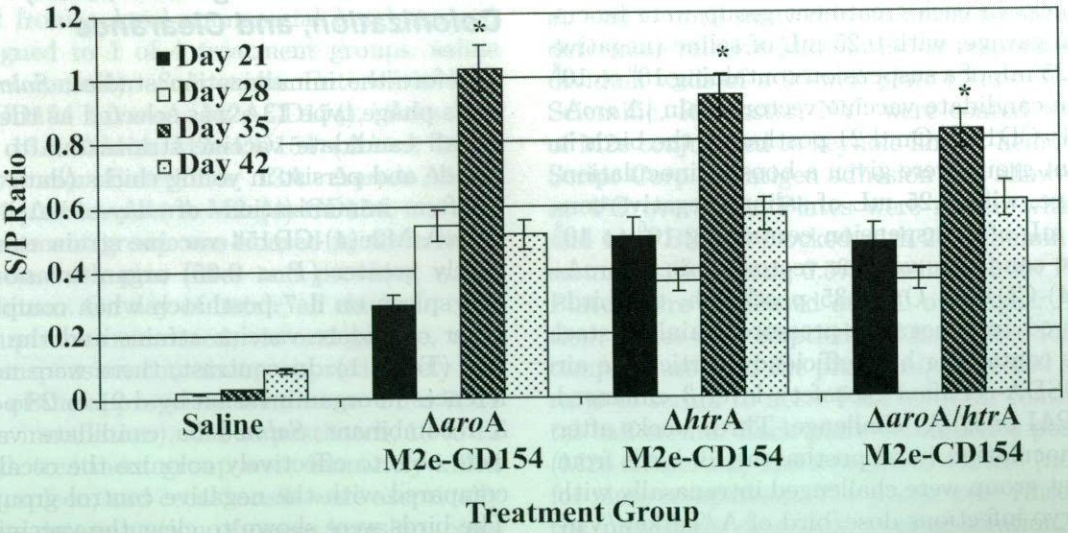

Figure 1. The M2e serum antibody response in commercial Leghorn chicks after oral immunization with live attenuated recombinant *Salmonella* vaccine vectors expressing M2e-CD154. Values are means ± SEM, representing 15 birds/treatment group. Chicks were orally inoculated with approximately 10^6 to 10^8 cfu of the appropriate treatment on day of hatch and 21 d posthatch. On d 21 (before booster immunization), 28, 35, and 42 posthatch, serum was collected and used in an antigen capture ELISA to determine relative M2e antibody responses. Antibody responses are represented as sample:positive control (S/P) ratios that were calculated using the following S/P mean ratio calculation: (sample mean − negative control mean):(positive control mean − negative control mean). *Means within a day with an asterisk indicate a significant difference ($P < 0.05$) between treatments.

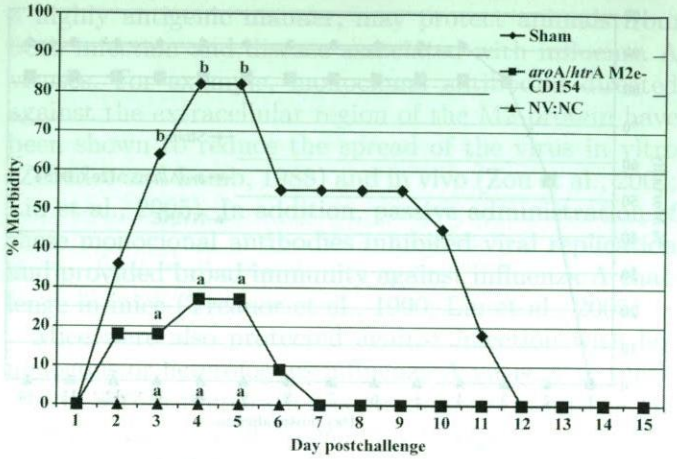

Figure 2. Morbidity of specific-pathogen-free Leghorns immunized with a live attenuated recombinant *Salmonella* vaccine vector expressing M2e-CD154 or saline after direct challenge with low pathogenic avian influenza (LPAI) H7N2. Data points represent the percentage of birds per treatment group exhibiting clinical signs of morbidity. Specific-pathogen-free Leghorn chickens were orally inoculated with 0.25 mL of saline or suspension containing approximately 10^6 to 10^8 cfu/mL of Δ*aro*A-*htr*A M2e(4)-CD154 on day of hatch and 21 d posthatch. Three weeks after the booster inoculation (42 d posthatch), all birds were challenged intranasally with 10^6 50% embryo infectious dose/bird of A/Turkey/Virginia/158512/2002 LPAI. NV:NC = nonvaccinated:nonchallenged.

es increased to 6.6, 6.3, and 6.3 for the Δ*aro*A M2e-CD154, Δ*htr*A M2e-CD154, and *aro*A-Δ*htr*A M2e(4)-CD154 strains, respectively.

Protection Against Direct AI Challenge

To investigate whether recombinant *Salmonella* expressing M2e could protect in vivo against AI challenge, we tested the *aro*A-Δ*htr*A M2e(4)-CD154 vaccine candidate against both LPAI and HPAI in chickens. After vaccination and direct challenge with LPAI H7N2, M2e-vaccinated birds exhibited significantly less morbidity compared with the sham (saline) group (Figure 2). Morbidity was highest at d 4 and 5 postchallenge in the M2e-vaccinated birds, with 27% of birds displaying clinical signs of disease including mild respiratory distress, depression, and ruffled feathers. Duration of morbidity in M2e-vaccinated birds ended on d 6 postchallenge. In contrast, sham (saline)-vaccinated birds displayed 82% morbidity on d 4 and 5 postchallenge, which also included respiratory signs, depression, and ruffled feather. Duration of clinical signs of disease lasted 11 d postchallenge.

Birds vaccinated with the candidate recombinant *Salmonella* vaccine strain also exhibited less incidence of viral shedding of the LPAI H7N2 virus as determined in both cloacal and oral swabs (Table 3). Significantly less incidence of shedding was observed in oral swabs on d 2 postchallenge in the M2e-vaccinated-challenged group versus sham-vaccinated-challenged group. No significant differences were observed in oral or cloacal swabs taken at d 4 postchallenge.

After direct challenge with HPAI H5N1, 55% mortality was observed in both vaccinated and sham-vaccinated birds at d 2 postchallenge (Figure 3). Greater than 80% of the *aro*A-Δ*htr*A M2e(4)-CD154 and sham-vaccinated birds were dead within 3 d. No protection after HPAI challenge was observed between the M2e and sham-vaccinated groups. After d 1, both challenged groups (sham-vaccinated-challenged and vaccinated-challenged) had increased mortality compared to the sham-vaccinated-sham-vaccinated group. No difference in incidence of viral shedding was observed in either oral or cloacal swabs (Table 3).

DISCUSSION

Current vaccination programs for poultry involve the use of conventional inactivated vaccines generated from amplified wild-type or recombinant fowlpox-vectored vaccines expressing an HA antigen (Swayne et al., 2000; Swayne and Suarez, 2000; Capua and Alexander, 2006; Swayne and Kapczynski, 2008b). A major advantage of inactivated killed vaccines lies in the ability to quickly change vaccine formulation as the field virus changes. However, these vaccines are relatively antigen-intensive,

Table 2. Ability of sera from commercial Leghorns immunized with live attenuated recombinant *Salmonella* vaccine vectors expressing M2e-CD154 to neutralize avian influenza (AI) in vitro[1]

	Neutralizing index[2]			
Treatment	21 d[3]	28 d	35 d	42 d
Positive control[4]	7.8	8.8	8.8	8.8
Δ*aro*A M2e-CD154	5.8	6.6	6.3	6.3
Δ*htr*A M2e-CD154	6	6.3	6.3	6.3
Δ*aro*A-*htr*A M2e(4)-CD154	5.8	6.3	6.3	6.3

[1]Sera samples were collected from 15 birds per treatment group on d 21, 28, 35, and 42 posthatch. The samples were pooled and sent to an independent laboratory (Charles River Laboratories Inc., Wilmington, MA) for analysis.

[2]Neutralizing index is expressed as the reciprocal dilution (2-fold) of serum required to inhibit cytopathic effect. Titers greater than 2 were considered to be positive and values equal to or greater than 7 were considered to be protective against the reference AI strain (H9N2 low pathogenic AI).

[3]Sera samples were obtained before booster immunization.

[4]Positive control was sera from Leghorn chickens that were hyperimmunized with synthetic M2e peptide conjugated to keyhole limpet hemocyanin (Genscript Corp., Piscataway, NJ).

have comparatively short-lived immunity, and must be administered by injection, causing considerable expense and reluctance for widespread adoption in commercial poultry (Swayne, 2003; Bublot et al., 2005). In addition, these vaccines also eliminate the ability to combine testing with vaccination using a differentiating infected from vaccinated animals (**DIVA**) approach (Savill et al., 2006). Similarly, the fowlpox-vectored approach requires manual administration at the hatchery but has the advantage of relatively long-lived immunity and the potential to simultaneously test within a DIVA strategy vaccination program (Swayne, 2003; Bublot et al., 2005). A major disadvantage of each of these approaches for widespread use in the commercial poultry industry is the cost of vaccine production and administration, likely limiting the use of these vaccines to high-risk areas and only temporary use. Therefore, a vaccine that is orally effective, inexpensive to amplify, and allows DIVA strategy testing in vaccinated flocks would prove advantageous over currently licensed products.

Oral live attenuated *Salmonella* vaccine vectors expressing recombinant foreign antigens have previously been shown to stimulate systemic, mucosal, humoral, and cell-mediated immune responses against *Salmonella* and foreign antigens (Mollenkopf et al., 2001; Kotton and Hohmann, 2004; Ashby et al., 2005), and attenuated strains of *Salmonella* have long been approved for use in human and veterinary medicine (Hormaeche and Khan, 1996). *Salmonella* vectors also have the potential advantage of being extremely inexpensive to amplify-manufacture and because they do not have to be injected and can be administered by spray or drinking water, they are much more acceptable for widespread administration to commercial poultry. The present study demonstrates that a live attenuated *Salmonella* vaccine vector expressing a conserved region of the M2e protein of influenza A viruses in combination with a biologically active region of CD154, when administered orally, is effective at eliciting an M2e-specific IgG antibody response and this alone provided protection against direct LPAI, but not HPAI, challenge.

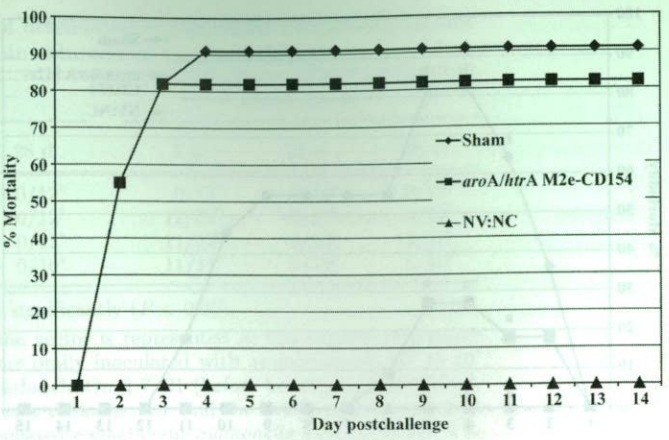

Figure 3. Mortality of specific-pathogen-free Leghorns immunized with a live attenuated recombinant *Salmonella* vaccine vector expressing M2e-CD154 or saline after direct challenge with high pathogenic avian influenza (HPAI) H5N1. Data points represent the percentage of birds per treatment group exhibiting clinical signs of morbidity. Specific-pathogen-free Leghorn chickens were orally inoculated with 0.25 mL of saline or suspension containing approximately 10^6 to 10^8 cfu/mL of Δ*aro*A-*htr*A M2e(4)-CD154 on day of hatch and 21 d posthatch. Three weeks after the booster inoculation (42 d posthatch), all birds were challenged intranasally with 100 50% chicken lethal dose (A/Egret/Hong Kong/757.2/2002) of H5N1 HPAI per bird. NV:NC = nonvaccinated:nonchallenged.

The M2e peptide consists of only 23 amino acid residues and, when presented by itself, is weakly immunogenic (Black et al., 1993; Fiers et al., 2004). However, attachment of this M2e peptide to an appropriate carrier can render it strongly immunogenic and several lines of evidence suggest that M2e, when presented in

Table 3. Viral shedding from chickens immunized with a live attenuated recombinant *Salmonella* vaccine vector expressing M2e-CD154 after direct challenge with avian influenza (AI)[1]

		Group,[2] no. positive/no. tested		
Virus challenge	Swab	NV/NC	NV/C	V/C
H7N2 LPAI				
Day 2 PC	Oral	$0/11^{a}$	$10/11^{b}$	$4/11^{a}$
	Cloacal	$0/11^{a}$	$5/11^{b}$	$2/11^{ab}$
Day 4 PC	Oral	$0/11^{a}$	$7/11^{b}$	$2/11^{ab}$
	Cloacal	$0/11^{a}$	$5/11^{b}$	$2/11^{ab}$
H5N1 HPAI				
Day 2 PC	Oral	$0/11^{a}$	$11/11^{b}$	$7/11^{b}$
	Cloacal	$0/11^{a}$	$9/11^{b}$	$7/11^{b}$
Day 4 PC	Oral	$0/11^{ND}$	0/2	1/1
	Cloacal	$0/11^{ND}$	0/2	1/1

[a,b]Rows with different lowercase superscripts indicate significant ($P < 0.05$) difference in incidence of viral shedding per group by Fisher's exact test. ND = no difference between groups.

[1]Specific-pathogen-free Leghorn chickens were orally inoculated with 0.25 mL of saline or suspension containing approximately 10^6 to 10^8 cfu/mL of *Salmonella* Enteriditis 13A Δ*aro*A-Δ*htr*A M2e(4)-CD154 on day of hatch and 21 d posthatch. Three weeks after the booster inoculation (42 d posthatch), all birds were challenged intranasally with either 10^6 50% embryo infectious dose of A/Turkey/Virginia/158512/2002 H7N2 low pathogenic AI (LPAI) or 100 50% chicken lethal dose of A/Egret/Hong Kong/757.2/2002 H5N1 high pathogenic AI (HPAI) per bird. Oral and cloacal swabs were taken on d 2 and 4 postchallenge (PC).

[2]NV/NC = nonvaccinated/nonchallenged; NV/C = nonvaccinated/challenged; V/C = vaccinated/challenged.

a highly antigenic manner, may protect animals from both infection and disease associated with influenza A viruses. For example, monoclonal antibodies directed against the extracellular region of the M2 protein have been shown to reduce the spread of the virus in vitro (Zebedee and Lamb, 1988) and in vivo (Zou et al., 2004; Liu et al., 2005). In addition, passive administration of these monoclonal antibodies inhibited viral replication and provided broad immunity against influenza A challenge in mice (Treanor et al., 1990; Liu et al., 2005).

Mice were also protected against infection with homologous or heterologous influenza A virus after vaccination with a preparation containing the complete M2 protein expressed in *Spodoptera frugiperda* cells (Slepushkin et al., 1995). In those experiments, the protein was partially purified, and the preparation was administered to mice with adjuvant. Nevertheless, mucosal administration of the M2e antigen linked to a vector virus has been shown to be completely protective in mice (Neirynck et al., 1999; Fiers et al., 2004; De Filette et al., 2005, 2006), partially due to the stimulation of mucosal immunity. It is widely accepted that mucosal exposure and generation of mucosal immunity may be necessary to provide maximal protection against mucosal pathogens, and that gastrointestinal exposure through vectored vaccines often confers protection against other mucosal (e.g., respiratory) pathogens exhibiting those epitopes (Holmgren et al., 1992).

The expected results of the implementation of a vaccination policy on the dynamics of infection are primarily those of reducing susceptibility to infection and reducing the amount of virus shed into the environment. From experimental data, it is known that efficacious LPAI and HPAI vaccines protect against clinical signs and mortality, reduce virus shedding, and increase resistance to infection (Capua and Marangon, 2004). Results from the studies presented here clearly show strong humoral response and protection after LPAI challenge, with decreased viral shedding. Although protection from direct challenge with HPAI was not observed by immunization with the M2e peptide with CD154 alone, future research will utilize other AI epitopes as a potential means to induce protective immunity against all AI serotypes.

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